Recent Developments in the Investigation of Thyroid Regulation and Thyroid Carcinogenesis

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This review covers new mechanistic information spanning the past 10 years relevant to normal and abnormal thyroid growth and function that may assist in the risk assessment of chemicals inducing thyroid follicular cell neoplasia. Recent studies have shown that thyroid regulation occurs via a complex interactive network mediated through several different messenger systems. Increased thyroid-stimulating hormone (TSH) levels activate the signal transduction pathways to stimulate growth and differentiation of the follicular cell. The important role of TSH in growth as well as in function helps to explain how disruptions in the thyroid–pituitary axis may influence thyroid neoplasia in rodents. New investigations that couple mechanistic studies with information from animal cancer bioassays (e.g., sulfamethazine studies) confirm the linkage between prolonged disruption of the thyroid–pituitary axis and thyroid neoplasia. New initiation/promotion studies in rodents also support the concept that chronic stimulation of the thyroid induced by goitrogens can result in thyroid tumors. Some of these studies confirm previous suggestions regarding the importance of chemically induced thyroid peroxidase inhibition and the inhibition of 3,3′,5,5′-tetraiodothyronine (T_4, thyroxine) deiodinases on disruption of the thyroid–pituitary axis leading to thyroid neoplasia. Some comparative physiologic and mechanistic data highlight certain differences between rodents and humans that could be expected to confer an increased vulnerability of rodents to chronic hypersecretion of TSH. New data from epidemiologic and molecular genetic studies in humans contribute further to an understanding of thyroid neoplasia. Acute exposure to ionizing radiation, especially in childhood, remains the only verified cause of thyroid carcinogenesis in humans. Iodine deficiency studies as a whole remain inconclusive, even though several new studies in humans examine the role of dietary iodine deficiency in thyroid cancer. Specific alterations in gene expression have been identified in human thyroid neoplasia, linked to tumor phenotype, and thus oncogene activation and tumor-suppressor gene inactivation may also be factors in the development and progression of thyroid cancer in humans. An analysis by the U.S. EPA Risk Assessment Forum, prepared as a draft report in 1988 and completed in 1997, focused on the use of a threshold for risk assessment of thyroid follicular tumors. New studies, involving several chemicals, provide further support that there will be no antithyroid activity until critical intracellular concentrations are reached. Thus, for chemically induced thyroid neoplasia linked to disruptions in the thyroid–pituitary axis, a practical threshold for thyroid cancer would be expected. More information on thyroid autoregulation, the role of oncogene mutations and growth factors, and studies directly linking persistently high TSH levels with the sequential cellular development of thyroid follicular cell neoplasia would provide further confirmation. *Key words*: growth factors, signal transduction, thyroid adenoma, thyroid carcinogens, thyroid carcinoma, thyroid hormone metabolism, thyroid peroxidase, thyroid-stimulating hormone. *Environ Health Perspect* 106:427–436 (1998). [Online 24 June 1998] *http://ehpnet1.niehs.nih.gov/docs/1998/106p427-436hard/abstract.html*

Most risk assessment issues involving the thyroid concern the role of prolonged elevation of circulating thyroid-stimulating hormone (TSH) levels in the development of follicular cell neoplasia in laboratory animals and the appropriate procedures for extrapolation of these results to humans. A Technical Panel of the U.S. EPA Risk Assessment Forum examined this issue and concluded in a 1988 draft report that, under certain circumstances, thyroid follicular cell tumors develop through linked steps beginning with interference in thyroid–pituitary status. When there is no direct interaction of the chemical with DNA, the EPA Technical Panel concluded that thyroid follicular neoplasia involves a nonlinear dose–response process and would not develop unless there is prolonged interference with the thyroid–pituitary feedback mechanisms. The mechanistic information assembled in the 1988 draft was published in 1989 (*1*). Into 1997, the EPA Risk Assessment Forum has continued to develop a science policy statement for assessing risk of thyroid follicular cell neoplasia, requiring an update of the pertinent literature as part of that process.

Since the EPA report on thyroid follicular cell carcinogenesis was published (*1*), more than 600 papers on thyroid function, regulation, carcinogenesis, and epidemiology have appeared in the literature. Recent studies on regulation of the thyroid gland and thyroid follicular cell neoplasia present a broad array of new data, which add depth and complexity to the information available in 1988 on this fundamental biological process. These studies provide information on growth factors and messenger systems, the neuroendocrine control of TSH secretion, the intrinsic heterogeneity in follicular cell populations with regard to proliferative potential, and new data on thyroid cancer in both rodents and humans, including molecular genetic and cytogenetic aspects. This review of the new publications highlights selected information that is considered most relevant to the mechanisms of normal and abnormal thyroid growth and function and the action of chemicals thereon.

Thyroid Regulation

Numerous recent studies point to the conclusion that the physiological regulation of thyroid cell growth and function involves a complex interactive network of trophic factors (endocrine, paracrine, and autocrine). The effects of these factors are mediated through a number of different second messenger systems. It is well established that TSH is the main growth factor for thyroid cells, maintaining the differentiated state of the thyroid and controlling thyroid hormone secretion. Other growth regulators involved in the complex web include insulin/insulinlike growth factor-I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGF-β), and an endogenous iodide dependent mechanism (*2,3*).

TSH exerts its action on thyroid follicular cells via receptor sites, restricted mainly to the basal membranes of follicle cells (*4*). The advent of recombinant DNA technology has led to the cloning of the TSH receptor of both rat (*5*) and human thyroid (*6–9*). The

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TSH receptor is a plasma membrane site able to bind G (guanine nucleotide-binding) protein for signal transduction (*8*). G protein activation by the TSH receptor appears to be a highly complex effector system involving all four G protein families (*10*). The gene for the TSH receptor is virtually constitutive in the thyroid cell, occurring far along the pathway of transformation, as demonstrated by persistent expression in normal thyroid tissue as well as in differentiated thyroid tumors, but not undifferentiated carcinoma (*11*). Current models indicate that the human TSH receptor is a heptahelical glycoprotein molecule with an extremely large extracellular domain at the *N*-terminus, a transmembrane/intracellular region consisting of seven intramembrane helices connected by three alternating intracellular and extracellular loops, and an intracellular tail at the carboxyl terminus (*12–14*). The extracellular domain is the ligand-binding site, its amino acid sequence and structure conferring a high specificity for recognition and binding of TSH, distinguishing it from other G protein-coupled receptors. It is thought that the three extracellular loops help the ligand to fit to the tertiary structure, while the intramembrane and intracellular segments appear to be critical for signal transduction. Available evidence indicates that the overall conformation of the TSH receptor in rats is probably the same as in humans, there being about 90% homology in the amino acid sequence of the rat and human receptors (*6,7,9*).

TSH, through activation of its receptor, has been shown to stimulate more than one signal transduction pathway in the regulation of both growth and differentiated function. Each pathway may be related to specific cellular events. The main effector of TSH on proliferation and differentiation in a variety of species, humans and rats included, is the cAMP signal transduction pathway, that is, the cascade involving activation of adenylate cyclase resulting in cAMP generation (*15,16*). The binding of TSH to its receptor is believed to produce a structural change in the receptor, activating the G protein to which it is coupled. Activation involves dissociation of the α_s subunit of the G protein ($G_s\alpha$), in turn stimulating adenylate cyclase, thus effecting a rise in cAMP (*14*).

There is increasing evidence that the physiological stimulation of thyroid cell function by TSH is achieved as well by the phosphatidyl-inositol/Ca^{2+} (Pi-C) signal cascade (via a G protein) with activation of phospholipase C in rat and human thyroid cells (*16–19*). [As a species difference, this apparently does not apply to dog thyroid cells (*20*)]. Thus the TSH receptor can activate both signaling systems, but with a different efficacy because, in contrast to the cAMP pathway, much higher concentrations of TSH are required to stimulate the Pi-C phospholipase C cascade (*17*). The signaling by these diverse pathways results in a range of metabolic consequences including iodine uptake and release, thyroid peroxidase generation, organification of residues on thyroglobulin, thyroid hormone synthesis and release, and thyroid cell growth and division (*16*). In this complex regulatory network, the TSH-cAMP cascade is functionally responsible for secretion, while the Pi-C phospholipase C cascade controls H_2O_2 generation and thyroid hormone synthesis (*21*). It is generally accepted that cAMP accounts for the mitogenic effect of TSH, mediated by the activation of cAMP-dependent protein kinases (*22*), although higher concentrations of TSH and more prolonged stimulation of the cAMP cascade are necessary to induce cell proliferation than for expression of differentiated function (*23*). cAMP also appears to play a central role in iodide uptake and metabolism by the follicular cell (*24,25*) and in thyroglobulin and thyroid peroxidase (TPO) gene expression (*26,27*).

A third distinct pathway for signal transduction related to cell growth and proliferation in the thyroid is the hormone-receptor–tyrosine protein kinase pathway. Receptors for certain growth factors, such as EGF and IGF-1, possess an intrinsic protein tyrosine kinase activity through which those growth factors appear to act (*2,3,15,28*). However, for the thyroid follicular cell this signal transduction cascade has not been well defined.

Growth factors also play a key role, along with TSH, in the complex regulation of thyroid cell proliferation, but there are few data yet to explain how these trophic factors interact with the cell cycle to stimulate or inhibit cell division in the thyroid. There is evidence from the rat cell line FRTL-5 that both TSH receptor gene expression and thyroglobulin gene expression are under the control of insulin/IGF-1 at a transcriptional level as well as TSH (*29–31*). Similar evidence for a complex autoregulatory feedback mechanism involving insulin/IGF-1 operative at several levels of interactive signaling is accumulating for other primary thyroid cell culture systems (*32,33*). Collective data suggest that a complex interaction between the 1,2-diacylglycerol/protein kinase C (one of the bifurcating second messenger pathways of the Pi-C signal cascade) and the adenyl cyclase signal transduction systems is important in the regulation of thyroid growth by TSH and IGF-1 (*34*). Thus, in rats, dogs, and humans, insulin/IGF-1 is considered a necessary cofactor for the action of TSH on follicle cells, synergizing with TSH to induce thyrocyte proliferation while maintaining differentiated function (*3,35*). In humans, it has been recorded that benign and malignant thyroid tumors produce increased levels of IGF-1 (*36,37*), leading to the suggestion that emergent adenoma cells lose their dependence on exogenous IGF-1 and acquire the capability for autocrine production of this growth factor. This could result in continued autostimulation of cell replication, allowing thyroid nodules to become autonomous (*38,39*).

Other autocrine/paracrine regulators of thyroid growth, with potent mitogenic activity for thyroid cells demonstrable *in vitro*, include EGF and bFGF. EGF is synthesized within the thyroid gland and induces proliferation in thyroid cells from a wide range of species at the expense of dedifferentiation and loss of specialized thyroid-specific function (*40,41*). bFGF is present in human thyroid tissue (*42*), and there are stores of FGF in the basement membrane of follicles in normal adult rat thyroid (*43*). EGF has been shown to stimulate the growth and invasion of differentiated human thyroid cancer cells in culture and in nude mice (*44*), whereas bFGF expression increases during thyroid hyperplasia in the rat (*45*).

Transforming growth factor β_1 (TGF-β_1) is a putative negative regulator of thyroid growth, as studies in normal cell systems have shown it to inhibit thyrocyte proliferation, including that mediated by TSH (*46–48*). Although the actual role of TGF-β_1 in the thyroid is not known, cell culture and rodent investigations suggest that it is a limiting autocrine influence on thyroid cell hyperplasia and cancer growth (*49,50*). In one study, TGF-β_1 was detected in approximately 50% of human thyroid carcinomas, but not in adenomas, with a striking correlation being observed between the dual presence of TGF-β_1 expression and arginine substitution at codon 61 of the H-*ras* oncogene (*51*). Lazzereschi et al. (*52*) demonstrated a significant reduction of TGF-β receptor type II mRNA and protein expression in thyroid carcinomas but found no reduction in thyroid adenomas. These studies suggest that escape from the growth-inhibitory effect of TGF-β may play an important role in the progression of human thyroid cancer.

There has also been additional evidence that iodine is a major mediator of thyroid autoregulation, involving numerous inhibitory actions (*53*). One of these is a decrease in cAMP formation in response to TSH, resulting in an inhibition of all

cAMP-mediated stimulatory effects of TSH on the thyroid. Excess iodide therefore exerts a negative control on different thyroid parameters, inhibiting iodide uptake and organification, protein and RNA biosynthesis, hormone secretion, as well as paracrine mitogenic activity on endothelial cells and fibroblasts (*54–56*). Most of these actions appear to be mediated by an intracellular organified iodine intermediate of unknown identity. Various derivatives of arachidonic acid have been proposed as this putative regulator(s), including the iodinated eicosanoid, δ-iodolactone (*56*). However, this compound had no effect on TSH-mediated cAMP formation in porcine follicles (*57*). Considered to be a more likely candidate is 2-iodohexadecanal (*58*), a major iodolipid formed in horse thyroid when incubated with iodide (*59*), but which is also detectable in the thyroid of other species, including humans and rats.

Transport, Metabolism, and Excretion of Thyroid Hormones

Recent studies have confirmed heterogeneity in the transport of thyroid hormones between species. In humans there are three main carrier proteins which reversibly bind circulating thyroid hormones, thyroxine-binding globulin being the major carrier protein for T_4. Transthyretin (formerly known as thyroxine-binding prealbumin) is a minor binding protein in humans, accounting for about 20% of circulating T_4, while albumin, through nonspecific binding, carries only 10% of the circulating hormone. Collectively these three proteins transport more than 95% of T_3 and T_4 hormones in humans (*60,61*). In rats, a significant difference is that thyroxine-binding globulin occurs only as a postnatal surge, declining to nondetectable levels by early maturity at 8 weeks, but reappearing in senescence (*62*). Thus, through most of their lives rats lack one of the major carrier proteins for T_4, with transthyretin serving as the primary plasma transporter. The binding affinity of transthyretin, however, is several orders of magnitude lower than for thyroxine-binding globulin (*63*). Likewise, T_3 is transported in humans in a bound state to thyroxine-binding gloubulin and albumin, but only by albumin in rodents (*63*). A primary function of the transport proteins is believed to be extrathyroidal storage of thyroid hormones as a mechanism to control hormone release, thereby protecting target tissues from excessive hormone influence (*60*). By virtue of the absence of a major high-affinity binding protein, this buffering action protecting targets such as the thyrotrophs of the anterior pituitary would appear to be lower in rodents. Furthermore, in species in which T_4 binding is limited to transthyretin and albumin, the proportion of unbound T_4 is greater than it is in humans (*63*). Coupled with a considerably shorter half-life for T_4 of 12–24 hr in rats compared to 5–9 days in humans (*63*), these various interspecies differences imply a greater predisposition of rodents to TSH perturbation by chemicals that influence thyroid status.

There has been much recent progress in understanding the enzymatic pathways responsible for metabolism of T_4, T_3, and the inactive T_3 analog, reverse T_3 (rT_3). T_4 is secreted by the thyroid but has little biological activity unless deiodinated to T_3. Two isoenzymes catalyze this 5′-deiodination reaction: type I 5′-deiodinase (a selenium-containing protein) abundant in liver, kidney, and thyroid, and type II 5′-deiodinase (lacking selenium), found primarily in brain, pituitary, and brown adipose tissue (*64,65*). In humans, about 80% of circulating T_3 derives from peripheral 5′-monodeiodination of T_4, particularly that by liver and kidney, while 20% of T_3 is secreted by the thyroid (*66*). In rats, the origin of circulating T_3 is more controversial. Based on studies with selenium-supplemented and selenium-depleted rats, Chanoine et al. (*65*) suggested that intrathyroidal conversion of T_4 to T_3 provides the major source of T_3 in this species. Comparatively, rat thyroidal levels of type I 5′-deiodinase are the highest so far reported for any species (*66*).

Recent studies have also confirmed that, besides deiodination, conjugations with either glucuronic acid or sulfate are significant metabolic pathways for thyroid hormones in rats (*67–69*). The enzymes responsible for glucuronidation of thyroid hormones are UDP-glucuronoysyltransferases (UDP-GT) located mainly in the endoplasmic reticulum of liver cells, but also found in intestines and kidney. It appears that there are at least three UDP-GT isoenzymes involved in rat liver. T_4 and rT_3 are glucuronidated by types I and II isoenzyme; T_3 is glucuronidated by the type III isoform (*70*). The T_3 glucuronide conjugate is excreted in bile, which may represent a reversible pathway as the conjugate is hydrolyzed by intestinal bacteria, creating an enterohepatic cycle enabling reabsorption of free T_3 (*67,71*). Evidence also suggests that there may be a more effective enterohepatic circulation in humans than in rats (*71*).

Sulfate conjugation of thyroid hormones is an alternative metabolic pathway that enhances enzymatic deiodination and facilitates their biliary and urinary excretion. The sulfate conjugate of T_3 is rapidly deiodinated by type I deiodinase through successive deiodinations of the tyrosyl (inner) and phenolic (outer) rings (*72*), thus releasing iodine into the circulation for reutilization by the thyroid (*73*). In humans, the majority of non-deiodinative disposal of T_3 occurs via this pathway (*74*).

Control of TSH Secretion in the Central Nervous System

At the central nervous system level, recent work has provided additional information on the control of TSH secretion by thyroid hormones in the anterior pituitary and via the hypothalamus. A discrete population of neurons synthesizing thyrotropin-releasing hormone (TRH), located in the paraventricular nucleus of the hypothalamus, is under negative feedback regulation by circulating thyroid hormones (*75,76*). Some results suggest that the biosynthesis of TRH is regulated by both T_3 and T_4 (*77*), although the mechanism by which T_4 plays an inhibitory role is unknown. The negative feedback of thyroid hormones on TSH secretion caused by antithyroid compounds appears to be exerted mainly at the pituitary level. This is because the increase in TRH release into hypophyseal portal blood produced by propylthiouracil (PTU) is relatively small (less than 50%) compared to the pronounced increase (up to 20 times at 3 weeks) in serum TSH (*78*). In humans, new highly sensitive immunometric assays used for measurement of TSH serum concentrations have underscored earlier work showing that thyroid hormone negative feedback on pituitary TSH secretion is mediated mainly by local generation of T_3 within the pituitary from T_4 by the 5′-deiodinase enzyme system (*79*). Although controversial, some data suggest that in rats serum T_3 has a greater inhibitory action on TSH secretion from the pituitary than does serum T_4 (*80*), at least in the euthyroid or mildly hypothyroid states.

Mitogenic Effect of TSH on Thyroid Tissue

Evidence concerning the mitogenic role of TSH for thyroid cells *in vivo* has been further consolidated over recent years. Studies from various laboratories using tritiated thymidine labeling, metaphase arrest techniques for mitotic index, or immunohistochemical decoration of statin (a non-proliferation-specific nuclear antigen identifying quiescent G_0-phase cells) show that TSH stimulates, in a dose- and time-dependent way, the recruitment of noncycling G_0 cells into the cycling compartment, as well as entry into S-phase and the entry of G_2 cells into mitosis (*81*).

Recent work has indicated that the normal rodent (and human) thyroid may have

an intrinsic heterogeneity in the follicular cell population regarding the capacity for proliferative response to TSH. One hypothesis suggests that there are a few subsets of stemlike follicular cells with a high growth potential compared to the majority of the population and that this trait is stable and heritable (*82–85*). According to this model, the clones of cells with extensive proliferative potential are the origin of the adenomas that develop under conditions of chronic TSH stimulation (*83,84*).

As proposed by Studer et al. (*86*), the above intrinsic stem cell clone model reconciles earlier kinetic observations (*87,88*) that hyperstimulated thyroid attains a plateau phase of growth or state of "desensitization," with the vast majority of follicular cells becoming refractory to the mitogenic effects of increased TSH levels before the emergence of adenomas. In this model, chronic TSH stimulation would select preexisting thyrocyte clones with the greatest proliferative potential and thus with the greater risk of neoplastic transformation. An alternative model for explaining the self-limited nature of hyperthyroidism and the development of hormone-responsive tumors in the chronically stimulated rat thyroid proposes that clones of cells escape from the desensitization mechanism through mutational events and natural selection, leading ultimately to tumor formation (*39*). Both models agree that thyroid carcinogenesis involves rare subsets of cells responsive to continued TSH stimulation but differ concerning the origin of follicular cell heterogeneity. Additional support for the intrinsic subset concept and/or the controlling influence of TSH on the development of selected clones of thyroid follicular cells comes from other studies on cell proliferation (*81*) and from those using transplantation methodology (*89–91*).

Rodent Thyroid Cancer Studies

Although additional bioassays have revealed new compounds with thyroid tumor-inducing capability in rodents, such as malonaldehyde (*92*), or have provided stronger evidence of tumorigenicity as in the case of sulfamethazine (*93,94*), the most significant studies in this area concern the promoting activity of antithyroid compounds. These studies have used *N*-bis(2-hydroxypropyl)-nitrosamine (DHPN) as the initiating agent. Promoting activity in the rat has been confirmed for many antithyroid compounds, including thiourea and potassium thiocyanate (*95*), PTU and potassium perchlorate (*96*), 4,4′-methylenebis(*N,N*-dimethylbenzenamine) (MDBA) (*97*), 2,4-diaminoanisole sulfate (*98*), sulfadimethoxine (*99*), and phenobarbital (*95,100*). Where thyroid gland and hormone parameters were measured, there were strong correlations between the tumor-promoting activity on the one hand and increased thyroid weight, decreased serum T_3 and T_4 levels, and increased circulating TSH on the other (*97,98,100*). One of these studies (with MDBA) also linked the increased circulating level of TSH with increased numbers of TSH-positive cells in the anterior lobe of the pituitary gland (*97*). The phenobarbital study was noteworthy in demonstrating a clear dose-dependent negation of thyroid tumor promotion and plasma TSH elevation by T_4 replacement therapy (*100*). In investigations where cell proliferative activity has been determined, the results strongly support an important role for high serum levels of TSH in the early stages of thyroid tumorigenesis (*99,101*).

These tumor promotion studies add further and consistent support to the hypothesis that antithyroid compounds exert effects secondarily on the thyroid through the chronic stimulation of persistently elevated levels of TSH.

Effects of Specific Chemicals on the Thyroid–Pituitary Axis

Several recent studies have provided more quantitative data on the time- and dose-dependent effects of specific antithyroid chemicals on thyroid hormone status, including PTU (*102*), sulfamethazine (*103*), and phenobarbital (*100,102*). These studies defined the effects as early but persistent decreases in circulating T_3 and T_4 levels and a substantial increase in circulating TSH. Particularly noteworthy are observations on sulfamethazine, for which the dose responsiveness for thyroid parameters in rats was studied with 10 dose levels spanning 3 orders of magnitude (*103*). Consistently, the parameters of thyroid weight and plasma T_3, T_4, and TSH levels displayed nonlinear dose–response curves with a major break in linearity from zero slope at around the 1,600 ppm dose level. These data suggested that, if coincident with tumor incidence data, a benchmark approach might conceivably be applied through the most sensitive indicator to provide a scientific basis for high- to low-dose extrapolation for secondary thyroid carcinogenesis.

There is also more detailed information available on the metabolic pathways and metabolites of such antithyroid compounds as methylmercaptoimidazole (MMI) and PTU (*104,105*). Some of these data confirm the importance of antithyroid drugs accumulating in the target organ and thus achieving effective intracellular concentrations at the intrathyroidal site of iodide organification as a requirement for antithyroid activity. Accordingly, it has been calculated that the intracellular concentration of PTU in the rat thyroid reaches approximately 20 μM (*106*), sufficiently high for iodination inhibition.

A particularly critical site of action representing a common intrathyroidal mechanism shared by PTU, ethylenethiourea (ETU), MMI, and aminotriazole (ATZ) is TPO inhibition. Antithyroid chemicals appear to be able to inactivate TPO in one of two ways, either by a reversible reaction that does not involve covalent binding, or by an irreversible interaction involving suicide inactivation of the enzyme. The latter reaction comprises branched pathways that proceed concurrently—namely, inactivation of the enzyme and turnover of the suicide substrate (*107*). Thus, suicide inhibitors inactivate TPO by covalent binding to the prosthetic heme group in the presence of H_2O_2, resulting in H_2O_2-dependent catalytic formation of reactive intermediates (*107,108*). In this case, *de novo* synthesis is required to restore the lost enzyme activity (*109*). The reaction between either PTU or ETU and the TPO/H_2O_2-generating system is reversible, representing metabolic detoxification of these compounds in thyroid and not suicide inactivation (*107–109*). This contrasts with the effects of MMI and other thiocarbamide goitrogens, as well as ATZ, which cause suicide inactivation of the enzyme via covalent binding (*107–109*). The difference in action between PTU or ETU and MMI, involving reversible inhibition of TPO on the one hand, and irreversible inactivation requiring *de novo* synthesis of enzyme to restore activity on the other, may account for the longer duration of effect and greater clinical potency of MMI (*109*). The different mechanism of TPO inactivation between compounds would not necessarily implicate a different overall mechanism of rodent thyroid carcinogenesis. However, the commonality of this intrathyroidal mechanism among antithyroid chemicals that appear to induce follicular cell carcinogenesis via secondary effects on the thyroid–pituitary axis suggests that TPO inactivation might be a useful additional criterion for categorizing these chemicals. It has been suggested further that, for risk assessment purposes, the effect on TPO provides a biochemical basis for the existence of a no observed effect level, or benchmark of effect, in chemically induced thyroid toxicity (*109*).

A marked species difference may exist between primates and rodents in the inhibition of TPO by antithyroid compounds. As examples, inhibition of monkey TPO requires approximately 50 and 450 times

the concentration of PTU and sulfamonomethoxine, respectively, than does rat TPO (*110*). This striking inequality might explain the greater susceptibility of the rat to the antithyroid effects of such compounds compared to the primate.

More recent data concerning the extrathyroidal action of antithyroid compounds on the peripheral conversion of T_4 and T_3 involving inhibition of T_4 deiodinases primarily in liver [reviewed by Curran and De Groot (*111*)] is available for PTU (*102*), ATZ (*112*), and phenobarbital (*102,113,114*). Unlike PTU, ATZ did not affect the outer ring 5′-deiodination pathway but stimulated inner-ring 5-deiodination of T_4, with a consequent increase in serum concentration of rT_3 (*112*), an inactive form of T_3. ATZ has no thiocarbonyl (or aromatic) group in its structure, and the lack of sulfur could be an explanation for the difference in peripheral action from PTU. The studies with phenobarbital have confirmed that this drug acts through an extrathyroidal mechanism by increasing both hepatic glucuronidation of T_4 as well as increasing the clearance of T_4 from serum (*102,113,114*).These effects were mediated by the substantial induction of the enzyme responsible for T_4 metabolism, as well as an increased biliary flow. The effect of phenobarbital solely on the peripheral pathway and the less potent consequence for thyroid hormone levels, compared to the effects of a centrally acting thiourylene compound like PTU, accords with its role as a promoter rather than an inducer of rodent thyroid tumorigenesis. Recent studies also support a conclusion that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) reduces thyroid hormone levels predominantly by an extrathyroidal mechanism involving potent induction of UDP-GT activity, resulting in increased hepatic T_4 glucuronidation (*115*). There is still no epidemiological evidence that chemicals such as phenobarbital, which affect thyroid function through a peripheral mechanism involving thyroid hormone metabolism, are associated with thyroid neoplasia in humans (*111,116*).

In keeping with an indirect action on TSH hypersecretion from the anterior pituitary, some antithyroid compounds have been shown to have central nervous system effects. Qualitative increases in TSH-producing thyrotrophs in the rat pituitary have been associated with administration of PTU (*117*) and 4,4′-oxydianiline (*118*), with a rapid reversal of morphologic changes upon cessation of treatment in the case of PTU. In the PTU study there was also a coincident decline in growth hormone cells, suggesting transdifferentiation of somatotrophs into thyrotrophs (*119*) and implying changes in cell type rather than in total cell number in the hypothyroid state. At the molecular level, this accords with the induction of increased levels of TSH mRNA in the anterior pituitary by PTU and a concomitant fall in growth hormone mRNA (*120,121*). The profound effect on cytoplasmic TSH levels affected both TSH α and β subunits in a dose- and time-dependent manner (*117,120,122*), but the increases were relatively greater in TSH β than in the α subunit. T_3 replacement reversed these specific subunit changes (*117*). These various studies confirmed a direct influence of "thyroid status" on the regulation of pituitary hormones at a pretranslational level.

There is evidence that genotoxic chemicals able to induce thyroid cancer in rodents have different morphological and physiological effects from those of known goitrogens. Thus, DHPN induces rat thyroid tumors along a multistage pathway involving focal atypical hyperplasia (originating from single follicles) rather than diffuse follicular hyperplasia (*123*). Furthermore, DHPN and *N*-nitrosomethylurea (NMU) do not appear to influence the thyroid–pituitary axis during the induction of thyroid carcinogenesis because these compounds did not increase thyroid weights unrelated to tumor development, cause a persistent elevation of serum TSH levels, or cause changes in serum T_4 levels (*124–126*).

Collectively, these studies with a range of compounds strengthen the hypothesis that antithyroid agents in rodents act by secondarily causing sustained elevations in serum TSH levels associated with the development of thyroid carcinogenesis. They also highlight the differences in pertinent effects between antithyroid compounds and those rodent thyroid carcinogens that are directly DNA reactive.

Epidemiology and Etiology of Human Thyroid Cancer

In humans, tumor histology is important to the understanding of the etiology of thyroid cancer because different types appear to represent separate biological entities with different clinical and epidemiological features (*127,128*). The most frequent type is papillary carcinoma, accounting for approximately 60% of all thyroid cancers; follicular carcinoma represents about 20% (*129*). In Sweden, there are regional differences in the incidence of papillary and follicular types of thyroid cancer defined by iodine status, with iodine-deficient areas being associated with a higher risk of follicular cancer (*128*). There is also some evidence that the incidence rates of these histological entities may be changing. The data from one study reflects an increase for papillary thyroid cancer in Sweden since 1919, but a decline for follicular cancer in cohorts born since 1939 (*127*). Although residence in endemic goiter areas in Switzerland was linked to a modestly increased probability of developing thyroid cancer (*130*), overall there remains a general view that no convincing evidence has yet emerged to link environmental thyroid cancer with areas of iodine deficiency. Furthermore, the long-standing program of supplementation of food items with iodine in Sweden has not affected thyroid cancer trends in iodine-deficient or iodine-rich areas (*128*). Vegetables known to contain, or endogenously generate, thiocyanate have not been found to enhance the risk of thyroid cancer, but possibly exert a protective influence (*131,132*). A meta-analysis of four similarly designed case–control dietary studies conducted in high thyroid cancer areas of Switzerland and Italy revealed an association only with diets rich in starchy foods and fats, while raw ham and fish were protective (*133*).

The only verified cause of thyroid cancer in humans is exposure to ionizing radiation. This association has been established for X-radiation therapy (*134–137*) and for radioactive fallout (*138,139*). Of the several events exemplifying the latter, the Chernobyl nuclear power plant disaster provides the most striking correlation. Since 1990, a high incidence of childhood thyroid cancer has been recorded in the Republic of Belarus, affecting predominantly children that were younger than 1 year old at the time of the accident (*139*). Almost all of the cases have been papillary carcinomas with short latency (*140*), in keeping with the observation that radiation-associated thyroid tumors are predominantly of the papillary type (*129*). The most biologically significant isotopes released in the fallout were radioiodines, primarily ^{131}I, and consequently radioiodine has been accepted as the causative factor (*141*). This stands in marked contrast to the lack of evidence incriminating diagnostic or therapeutic doses of ^{131}I (*142,143*). As with the Chernobyl experience, age at the time of treatment with X-radiation therapy is also an important factor in thyroid cancer development; the 67-fold risk at 12 years mean age declines to nonsignificance at a mean age of 29 years (*144*). In contrast to the risk posed by high-level ionizing radiation, a well-designed Chinese study indicates that lifetime exposure to low-level environmental radiation, with an estimated cumulative dose of 9 cGy, is not a risk factor for human thyroid cancer (*145*).

Graves' disease, an autoimmune thyroid condition, is associated with the presence of circulating antibodies stimulating the TSH receptor (TSHR Ab) (*146,147*).

Whether this disease carries an increased risk of thyroid carcinoma has been controversial. Nevertheless, from the collective published reports, Mazzaferri (*148*) concluded that thyroid cancer incidence in surgically treated Graves' disease patients is between 5% and 10%. It is generally agreed that thyroid cancer occurring in Graves' disease is an aggressive form (*149*). There is strong support for a pathophysiological role of TSHR Ab rather than circulating TSH in thyroid cancer development associated with Graves' disease (*149,150*). This is because serum TSH levels are suppressed in hyperthyroid patients with thyroid cancer, while TSHR Ab's are present in most cases (*149*). Evidence that TSHR Ab are circulating autoantibodies to the TSH receptor comes from the finding that they, and monoclonal antibodies to the human TSH receptor in thyroid tissue, mimic many of the activities of TSH on thyroid cells (*146,149,151*). However, the role of TSH in mediating the growth of thyroid nodules in humans needs further clarification (*152*).

Changes in Gene Expression in Thyroid Carcinogenesis

To date, there have been numerous studies at the molecular level exploring the changes in gene expression that might accompany human thyroid carcinogenesis, involving a wide range of protooncogenes, oncogenes, tumor-suppressor genes, or gene protein products. Despite the technical evolution from transfection methodology to polymerase chain reaction amplification coupled with sequence-specific oligonucleotide hybridization or probing, some of the findings are discordant. For example, conflicting results have been obtained for the *erb* family of "nuclear" oncogenes, and for involvement of mutations of the TSH receptor or retinoblastoma (*Rb*) gene in thyroid tumor development. Other investigations have indicated clear evidence of no role in thyroid cancer for mutation of the *APC*, $p16^{INK4}$ or *nm23* genes, or for abnormal expression of the central regulating genes, *myc, myb, fos,* or *jun* (*2,3,153,154*).

There is general agreement emerging that some of the genetic changes observed can be correlated with tumor histotype and stage of tumor development. Point mutations of *ras* are the most frequent single genetic abnormalities found in human thyroid tumors. Occurring in about 50% of follicular cell tumors (*2,153,154*), these mutations are regarded as early molecular events in the development of this particular tumor type (*155–158*). Although all three *ras* family members have been involved (Ha-*ras*, Ki-*ras*, N-*ras*), the changes have been associated mainly with Ha-*ras* mutation, the most common mutation site being codon 61 with glutamine to arginine substitution (*156,157,159*). When comparing the data for thyroid cancer associated with areas of iodine sufficiency, one study found a higher rate of *ras* mutation in thyroid tumors from iodine-deficient areas (*157*). Also prevalent in follicular adenomas are *gsp* mutations. Of the approximately 20 different Gα subunits of G proteins, the only one found to be mutated in thyroid neoplasia has been $G_s\alpha$ (*160*). This mutation occurs in about 25% of cases of follicular adenoma, with a possible predilection for microfollicular adenomas (*3,154,161,162*). Such distribution suggests that *gsp* mutation is another early event in the development of follicular thyroid cancer. However, there are some discrepancies between studies concerning the involvement of G proteins in thyroid neoplasia (*163*), and mutations in $G_s\alpha$ have been found in only a minority of toxic thyroid adenomas (*164*).

In contrast to follicular tumors, an absence of *ras* and $G_s\alpha$ gene mutations has been recorded for papillary thyroid cancer (*165–166*). Instead, rearrangements of *ret* and *trk*, protooncogenes of the tyrosine protein kinase receptor family, are early events associated with the papillary type of cancer (*158*). The *ret*/PTC rearrangement is specific for the thyroid and is found in up to 30% of human papillary carcinomas (*154,167,168*). As this alteration has been detected in more than 40% of occult papillary carcinomas, generally considered to be the incipient stage of papillary malignancy, it is believed to represent an early event in the development of this tumor histotype (*168*). A higher prevalence of *ret* protooncogene rearrangement, in up to two- thirds of cases, has now been recorded in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear reactor accident (*169–171*). Unlike the situation in adult tumors and those associated with therapeutic irradiation (*172*), where the most common *ret* translocation is *ret*/PTC1, the alteration in the childhood radiation tumors from Belarus has been preferentially *ret*/PTC3 (*ELE/RET*) rearrangement (*169–171*), with several aberrant forms of fusion being described (*173*). Interestingly, the only reproducible cytogenetic abnormality found in papillary thyroid cancer has been an inversion of chromosome 10 at the 10q11.2 locus, which is known to involve the *ret* protoocogene at that locus (*174,175*). As one possible mechanism linking these changes with neoplastic transformation, expression of the *ret*/PTC1 oncogene has been shown to impair the activity of certain transcription factors involved in regulating differentiated functions of thyroid cells (*176*). Activating rearrangement of the *trk* protoocogene has also been found only in papillary carcinomas (*153,154*); overexpression of the *met* oncogene is another molecular aberration observed mainly in this tumor histotype (*3,154*).

Little is known concerning molecular changes involved in the transition from follicular cell adenoma to carcinoma, although a loss of heterozygosity involving chromosome 3p was considered to be specific for follicular carcinoma, appearing to correlate with the transition from the adenoma to the carcinoma stage (*177,178*). Chromosomal analysis of follicular thyroid tumors has also indicated the existence of three cytogenetically distinct subsets of adenoma, with numerical changes in chromosomes 5, 7, and 12 as the most frequent cluster of anomalies (*179*). A similar cluster of alterations found in some thyroid nodular hyperplasias has been interpreted as support for a biologic continuum between hyperplastic nodules and the most common subset of adenomas (*180*). At the histological level, polysomies for chromosomes 7 and/or 12 have been observed only in lesions with an exclusive or predominant microfollicular component (*181*). There is some evidence from several studies that mutation of the tumor-suppressor gene *p53* is a late genetic event in thyroid carcinogenesis involved in the progression to a more aggressive phenotype in the form of undifferentiated or anaplastic cancer (*165,182,183*). *In vitro* data suggest that progression to the anaplastic phenotype involves cooperative events of *p53* mutation together with dedifferentiation, an epigenetic event (*158*).

The role of TSH receptor gene mutations in the development of thyroid cancer is uncertain. Russo et al. (*184*) found activating mutations in the third intracellular loop of the TSH receptor in half of differentiated thyroid carcinomas constitutively expressing high adenylate cyclase activity. Other studies have found infrequent involvement of such genetic changes in malignant transformation of the thyroid, leading to the conclusion that mutational activation of intracellular domains of the TSH receptor is not an important mechanism of thyroid tumorigenesis (*185,186*). In contrast, somatic mutations of the TSH receptor gene are considered to be the most frequent cause of toxic thyroid adenomas, autonomous, frequently self-limiting, hyperfunctioning nodules that are not dependent on TSH (*164,187*). When introduced and expressed in thyroid cells, these mutations are able to confer TSH-independent growth, providing a specific molecular link between TSH receptor mutations and toxic adenomas (*188*). The majority of toxic adenomas with constitutively activating TSH receptor mutations appear to result from clonal expansion of a single cell (*189*).

Genetic alterations have also been detected in rat thyroid carcinogenesis, but only a few investigations have been reported. Ha-*ras* activation was exclusively involved in a majority of tumors induced by the direct-acting genotoxin NMU (*190*). In DHPN-induced rat thyroid tumors, however, mutations involved the Ki-*ras* gene via a G to A transition at the second base of codon 12 (*191*). The same point mutation was detected at an early time point in preneoplastic thyroid, suggesting that Ki-*ras* mutations may play a role in the development of DHPN-induced rodent thyroid cancer. In about half of cases, radiation-induced thyroid tumors in rats were associated preferentially with Ki-*ras* activation (*190*). In this respect the rat data conform with that for radiation-induced papillary thyroid tumors in humans, which also are associated with Ki-*ras* mutation (*192*). On the other hand, ATZ-induced adenomas in the rat showed only a low incidence of Ki-*ras* activation (*190*). In studies using a series of transplantable rat thyroid carcinoma cell lines induced by DHPN, Kitahori et al. (*193*) confirmed the activation of the Ki-*ras* gene as a frequent event but also found a point mutation of $G_s\alpha$ in several lines. Point mutations in the $G_s\alpha$ gene, fixing the $G_s\alpha$ protein in its active state, inhibit GTPase activity, thus increasing intracellular cAMP levels via stimulation of adenylate cyclase. In the same study, a point mutation in the intracellular domain of the TSH receptor gene was found in only one cell line, and there was an absence of *p53* mutations. The authors concluded that G to A point mutations in $G_s\alpha$ and *ras* genes directly reflected interaction of the carcinogenic nitrosamine with guanine residues in DNA (*193*).

Data Gaps and Research Needs

Much of the recent new data strengthen the hypothesis that nongenotoxic, antithyroid compounds induce rodent thyroid follicular cell carcinogenesis by an indirect mechanism involving thyroid–pituitary feedback regulation, and none of the data negate that notion. Some of the new evidence also supports the view that humans may be less sensitive to the antithyroid process than rodents. Nevertheless, there are gaps in the available information that require further research, as listed below.

- Despite the voluminous literature on thyroid autoregulation, more research is needed before the complex interactive network is fully elucidated.
- More data are required to clarify the role and interaction of oncogene mutations and growth factor alterations in thyroid carcinogenesis in both rodent and human, and particularly in defining differences, if any, between rodent thyroid cancer induced by antithyroid compounds compared to the action of genotoxic carcinogens.
- The differences that might distinguish the thyroid-related mechanisms of action of antithyroid compounds versus genotoxic carcinogens, such as DNA adduct analysis within the thyroid, and the effects of DNA-reactive carcinogens on TPO, require elucidation.
- More information is required directly linking persistently high levels of circulating TSH with a step-by-step sequence involved in the cellular development of thyroid follicular cell neoplasia.
- Well-conducted studies are needed to better define the comparative sensitivity of humans, relative to rodents, to the long-term effects of antithyroid factors.
- Using analytical epidemiology techniques, the environmental and dietary risk factors for thyroid cancer based on histological type in various human populations need to be better defined.

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